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5-Aminolevulinic Acid Induced Endogenous Porphyrin Fluorescence in 9L and C6 Brain Tumours and in the Normal Rat Brain

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Summary

A new approach in photodynamic therapy is the use of endogenous porphyrins for sensitisation of tumours to light. The induction of endogenous porphyrins after intravenous injection of 5-aminolevulinic acid (ALA, 200 mg kg^{-1}) was studied in 23 rats, bearing intracranial 9L or C6 tumours. After 0, 2, 4, 6, 8, and 22 hours the rats were sacrificed and the fluorescence distribution of endogenous porphyrins was studied in brain tissue sections with a standard fluorescence microscope and a confocal laser scanning microscope. The role of blood-brain barrier disruption on porphyrin production was studied in 2 rats with a cryo-lesion of the cortex.

Additionally, 9L and C6 tumour cell cultures were incubated with ALA for 8 hours in vitro. Fluorescence was measured with a fluorescence spectrophotometer in cell cultures and in the brain sections. Porphyrins were detected in vitro in the tumour cells from 2 hours onwards and ex vivo in the tumour sections mainly from 2 to 8 hours, by 22 hours porphyrin fluorescence had almost disappeared. The contralateral brain showed low fluorescence levels between 2 and 6 hours after ALA administration. At the site of the cryo-lesions low fluorescence was measured 6 hours after ALA administration.

The 9L tumours fluoresced homogeneously, with a sharp demarcation towards normal brain tissue. Fluorescence in the C6 tumours was patchy, with a poorly fluorescing edge. In both tumour models fluorescence was also detected in brain surrounding the tumour and sometimes in contralateral white matter and ventricle ependyma and pia mater. The slight increase of porphyrin fluorescence in the normal brain of tumour bearing rats, compared to the absence of this in rats without a tumour, was attributed to transport by bulk flow of porphyrins made in the tumours, and possibly also of circulating porphyrins or ALA leaking from the tumour vessels.

Keywords: Endogenous porphyrins; brain tumour photosensitizer; photodynamic therapy; 5-amino levulinic acid.

Introduction

Traditional photodynamic therapy (PDT) is a two-step treatment. A photosensitizer is administered that would ideally accumulate in the tumour, and subsequent irradiation with the appropriate (laser) light would then cause singlet oxygen mediated damage to the tumour cells. Of utmost importance for the treatment is a sufficient availability and selectivity in the retention of the photosensitizer in the tumour compared to the surrounding non-malignant tissue.

The clinically available photosensitizers Haematoporphyrin derivative (HpD) and Photofrin® do only show a minor, dose-dependent penetration into the normal brain after intravenous administration [7, 22, 54]. Due to a partial loss of blood-brain barrier (BBB) function, experimental [7, 22, 23, 29, 49, 53] and human [30, 37, 39, 54] brain tumours have been shown to contain considerable amounts of HpD after intravenous (i.v.) injection. However, the uptake and retention pattern is rather inhomogeneous after i.v. injection [6]. Therefore alternative ways of drug delivery were sought for PDT of brain tumours. One possibility is intratumoural injection to circumvent the BBB and to locally increase the photosensitizer concentration. While intratumoural delivery of photosensitizers can indeed increase the drug levels within the tumour [29, 49], insufficient distribution of the drug throughout the tumour and neurotoxicity to adjacent normal tissue, even without irradiation have been proven disadvantages of this mode of application [19, 36].

A new approach in PDT is the use of endogenous porphyrins [24]. Their synthesis can be stimulated through the administration of 5-aminolevulinic acid (ALA). As a result of external ALA administration, the tightly regulated intracellular concentration of endogenous porphyrins, mainly protoporphyrin IX (PpIX), is temporarily increased (Fig. 1). Depending

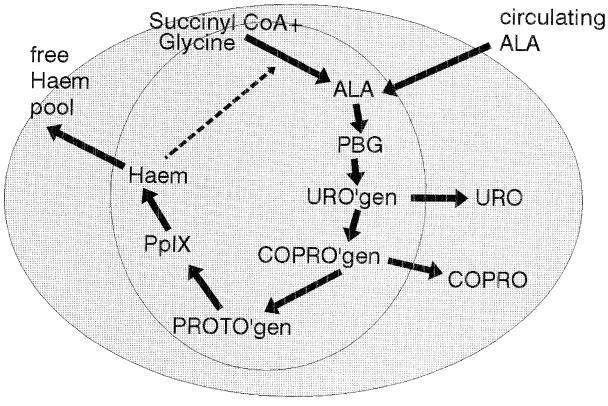

Fig. 1. Haem biosynthesis in the cell. The inner ellipse represents the mitochondria and the outer circle the cell membrane. *PBG* Porphobilinogen, *URO'gen* uroporphyrino-gen, *COPRO'gen* copro-porphyrinogen, *PROTO'gen* proto-porphyrinogen, *PpIX* proto-porphyrin IX

on the rate of metabolism of PpIX the tissue is prone to accumulate this endogenous photosensitizer during some time after ALA administration. In several neoplastic tissues there is evidence for an altered metabolism of haem, resulting in an increased temporary accumulation of porphyrins [11, 42, 56]. Selective PDT of oral and skin tumours based on this high difference in porphyrin level between tumour and normal tissue has been reported [17, 25].

The specific aim of this study was to investigate the possibility of selectively inducing endogenous porphyrins in two different rat brain tumour models in vitro and in vivo after ALA administration and to determine time kinetics of this process. We used fluorescence imaging for the localisation of the porphyrins in tissue sections, and a double ratio fluorescence technique to measure the amount of fluorescence in the tissue sections and in tumour cell cultures.

Materials and Methods

Tumour Models

The 9L-gliosarcoma cells were kindly provided by the Brain Tumor Research Center, University of California, San Francisco, USA. They were grown at 37°C in air with 5% CO_2 in Dulbecco's Minimal Essential Medium (Gibco Laboratories, Uxbridge, UK) with 10% FCS, 50 µg ml^{-1} streptomycin and 50 U ml^{-1} penicillin added. After trypsinization near confluent 9L cells of passage nr. 25 and 27 were adjusted to a concentration of 4×10^4 cells in 3 µl 0.9% NaCl for inoculation. They were injected stereotactically 5 mm deep into the caudate nucleus of 4 male Copenhagen rats (313–382 gr) and 10 male Fisher rats (275–335 gr, SPF, Harlan CPB, Zeist, Netherlands) as described before [19]. Frozen C6-glioma cells were kindly supplied by Dr. Oudeweernink (Dpt. of Enzymology, Rijksuniversiteit Utrecht, Netherlands). The cells were grown identically to the 9L cells. C6 cells of passage 50 and 51 were adjusted to 5×10^4 cells in 3 µl 0.9% NaCl. They were inoculated following the same procedure as with the 9L cells in 5 Copenhagen rats (312–351 gr) and 10 male Fisher rats (280–345 gr).

For the in vitro fluorescence spectra near confluent 9L and C6 cells were trypsinized and resolved in calcium containing phosphate buffered saline (PBS). The cell suspensions were gently stirred and placed in the fluorescence spectrophotometer. The porphyrin fluorescence was measured in 1 ml samples before, and every 30 to 60 min until 8 hours after administration of 200 µg ALA (Fine Tech Ltd., Haifa, Israel, and Janssen Pharmaceuticals, Tilburg, Netherlands) per ml cell suspension in PBS. Between the measurements the cells were kept in the dark at 37°C and before each measurement the suspension was stirred. The fluorescence measurements were performed according to the method described below for the tissue sections. Instead of a sample holder disposable plastic cuvettes were used for the cell suspensions.

Experimental Procedure

A total number of 37 animals were used, 29 with a tumour and 8 without. These animals were divided over 1 experimental group (23 animals with a C6 or 9L tumour) and 4 control groups (6 animals with a C6 or 9L tumour and 5, 2 and 1 animal without a tumour) (see table 1).

Two weeks after tumour implantation the 23 rats (group A) were briefly anaesthetised with Fluothane® (Zeneca BV., Ridderkerk, Netherlands) and received ALA by slow intraveneous injection of 200 mg kg^{-1} in 1 ml NaCl. Seven control rats without tumours (groups D and E) were also injected with ALA and were sacrificed 6 h later. Two of these (group E) had a cryo-lesion made to the cortex by applying a frozen iron rod (diameter 1 mm, −40°C) for 10 sec to the cortex through a burr hole in the skull, immediately prior to the ALA administration. Following ALA infusion the rats were housed in the near dark until they were sacrificed by cervical dislocation 0 to 22 h later, 3 to 5 animals at a time. The brains were removed and frozen immediately in pentane kept at −30°C and stored at −40°C until cutting on a cryostat. Six tumour bearing control rats (Group B) and one without a tumour (Group C) did not receive ALA.

Fluorescence Detection and Histology

Haematoxylin stained sections were used to compare the histological features of both tumour models. Unstained 20–40 µm sec-

Table 1. *Overview of the Various Groups. A: Main Experimental Group and B..E Control Groups*

Group	Treatment	No tumor	9L	C6
A	O hr ALA		0	2
	2 hr ALA		3	2
	4 hr ALA		1	2
	6 hr ALA		3	2
	8 hr ALA		1	2
	22 hr ALA		3	2
B	no ALA		3	3
C	no ALA	1		
D	6 hr after ALA	5		
E	cryo lesion +6 hr ALA	2		

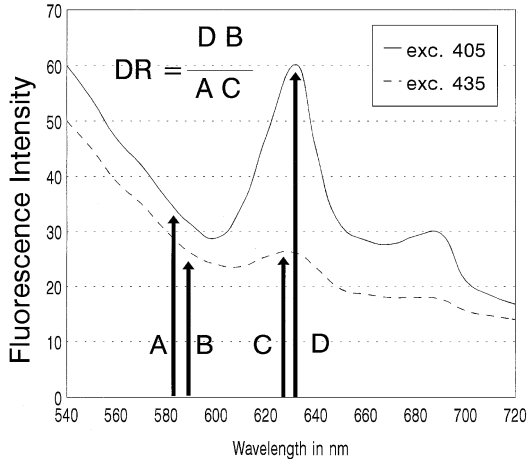

Fig. 2. Two fluorescence spectra are measured. One at 405 nm excitation wavelength and one at 435 nm excitation, the first producing a much stronger porphyrin peak. The fluorescence double ratio, *DR,* is calculated as indicated where A, B, C and D are indicated in the graph and stand for: $A\ F(405, \lambda_G), B\ F(435, \lambda_G), C\ F(435, \lambda_R), D\ F(405, \lambda_R)$

tions were examined by standard fluorescence microscopy (Olympus type AH2). For excitation a mercury lamp with a 405 nm bandpass filter was used and for the emission a long-pass filter transmitting wavelengths above 455 nm.

Double Ratio Fluorescence Spectrophotometry

A Perkin-Elmer fluorescence spectrophotometer (Perkin-Elmer, 650–40) was used to measure fluorescence emission spectra of dried 20 µm cryosections of tumour or brain. Two spectra were measured subsequently of each sample without moving it, one at 405 nm excitation, and the other at 435 nm excitation. From each spectrum two values of the fluorescence were determined graphically, one in the yellow, $\lambda_G = 580$ nm, and one in the red at the peak fluorescence of the porphyrin, λ_R (Fig. 2). From these numbers a double ratio, *DR*, was calculated according to:

$$DR = \frac{F_{(405,\lambda_R)} \times F_{(435,\lambda_G)}}{F_{(405,\lambda_G)} \times F_{(435,\lambda_R)}}$$

The virtue of the double ratio technique is that it produces a fluorescence value that only depends on the concentration of the fluorophore and that is not influenced by spatial or inter-sample variations in the optical absorption and scattering coefficients [45]:

$$DR = \frac{1 + aC}{1 + bC}$$

with a and b being constants ($a \gg b$) and C being the porphyrin concentration in the tissue. Moreover, it corrects for possible variations in the optical geometry induced by the variability in the position of the sample. At zero porphyrin concentration the double ratio will be 1. Calculation of the double ratio from the measured spectra is illustrated in (Fig. 2). For every tumour and every control three measurements were performed on different tissue samples. Measurements lasted approximately 1 minute. Within the period no photobleaching was observed.

Confocal Laser Scanning Fluorescence Microscopy and Image Analysis

Brain sections of 8 rats with either 9L or C6 tumours were studied 2, 4, 6, and 8 h after ALA administration by confocal laser scanning microscopy (CLSM). The CLSM (Zeiss) was operated in the laser confocal fluorescence mode. Fluorescence emissions were imaged through a 20× or 40× dry objective lens. The 512 × 512 raster laser scanning was carried out with a 20× or 40× electronic zoom. The fluorescence emission in the range of 620–640 nm was detected by photomultiplier tube. Each fluorescence image of any given field was constructed by adding 20 sequential optical images. Photomultiplier voltage and sensitivity (contrast and brightness and filters) were fixed for all fields and all slides imaged.

Statistics

For the comparison of different fluorescence means an independent *t*-test was used. Standard deviations are given if appropriate. *P* values smaller than 0.05 were considered statistically significant.

Results

Induction of Porphyrins in Tumour Cell Lines

In the cell suspensions the first porphyrin fluorescence could be detected starting at 2 h after incubation. The fluorescence of both cell solutions formed a broad band between 590 and 660 nm, with main peaks at 595, 625, 635 and 655 nm (Fig. 3a and 3b). The double ratio for these wavelengths is shown in time in (Fig. 4). An increase of the fluorescence with levelling of the fluorescence at 4 h after ALA administration is seen.

Induction of Porphyrins as Function of the Time in Brain Tumours

Intracranially grown tumours had a slightly different porphyrin fluorescence emission pattern. One major peak at 635 nm and a corresponding smaller peak at 705 nm were observed in both tumours (Fig. 5). In C6 tumours sometimes a separate 620 nm peak occurred but often only a slight shoulder could be observed at 620 in a much larger 635 nm peak (Fig. 5b).

Within the tumours the fluorescence signal showed large variations in intensity. In both tumour models the mean double ratio at 635 nm, $DR_{(635)}$, was increased from 2–8 h after injection of ALA, with a peak at 6 h (Fig. 6a). The double ratio at 635 nm, $DR_{(635)}$, was higher in 9L tumours than in C6 tumours, with the exception of 6 h after ALA administration. Twenty-two hours after ALA administration the fluorescence was still slightly elevated in the 9L tumours, while it had disappeared in C6 tumours.

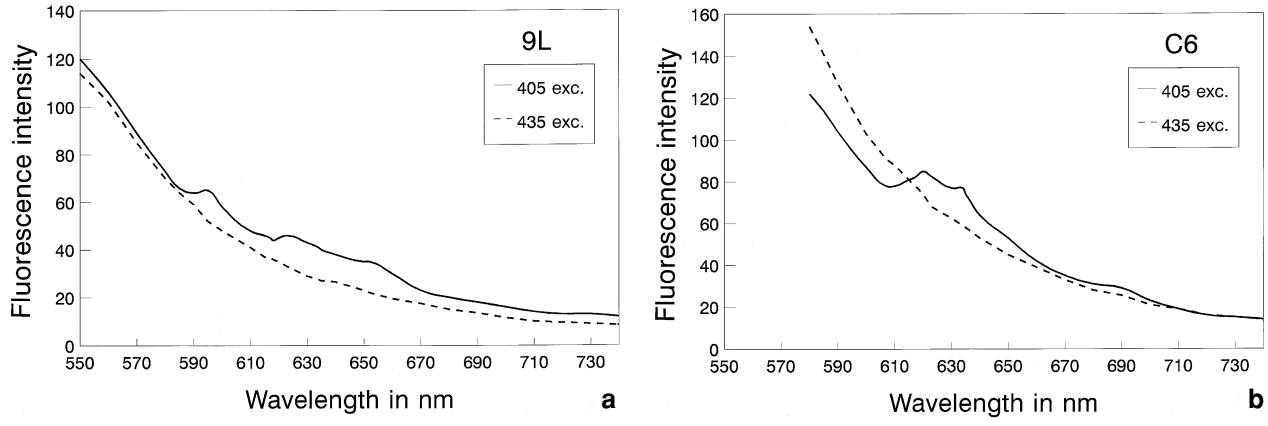

Fig. 3. Example of fluorescence emission spectra of tumour cell suspensions after 3.5 h incubation with 200 µg ALA ml^{-1}. (a) 9L gliosarcoma, (b) C6 glioma

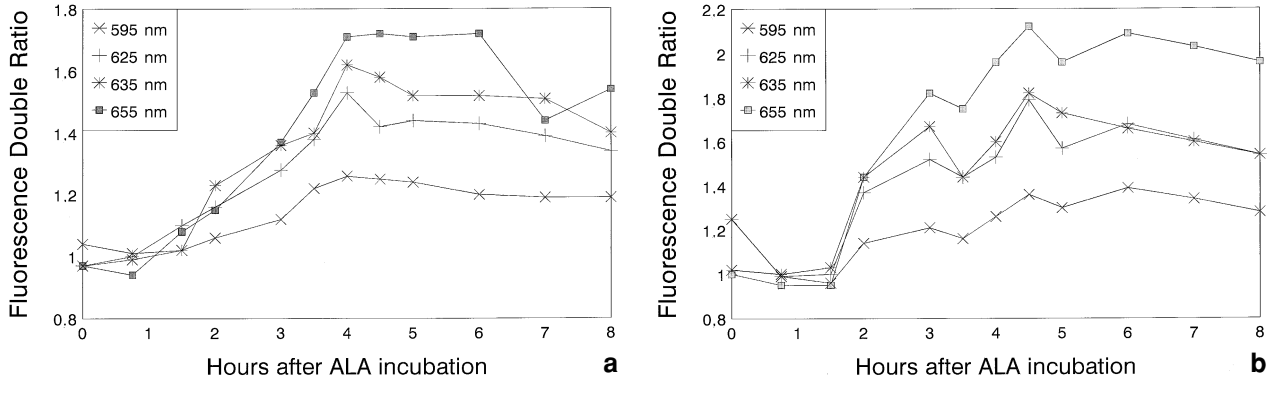

Fig. 4. The double ratio fluorescence calculated for different fluorescence maxima (595, 625, 635 and 655 nm) in (a) 9L and (b) C6 cell suspensions after ALA administration. Note that zero porphyrin concentration corresponds with a DR of 1

With standard fluorescence microscopy a diffuse, homogeneous, faint red fluorescence was detected in the 9L tumours 2 and 4 h after ALA administration. CLMS images gave an additional detail. The fluorescence was accentuated around blood vessels in the tumours (Fig. 7). At 6 h the fluorescence in the cytoplasm of the cells had turned bright throughout the tumours, with a pronounced fluorescence and sharp demarcation of the islands of perivascular tumour growth. At 8 h the fluorescence had diminished and at 22 h it had almost disappeared. The fluorescence picture for the C6 tumours deviated from that of the 9L tumours. Instead of a rather homogeneous fluorescence throughout the tumours, areas without fluorescence occurred throughout the tumours. Typically the necrotic parts, which were exclusively seen in C6 tumours, showed no fluorescence. In general the tumour parts growing over the brain surface, in the superficial cortex or deep in the striatum showed higher fluorescence intensity than those invading white matter. Contrasting with the strongly fluorescent infiltrations of the 9L tumours, in the C6 tumours the tumour edge, which was sharply demarcated, was often non-fluorescent. As in the 9L model, the C6 showed the strongest fluorescence after 6 h. After 22 h no fluorescence was detected in the C6 tumours.

Induction of Porphyrins in Normal Brain

In the normal striatum of the tumour bearing rats, contralateral to the tumours, a 10% increase of *DR* was measured between 2 to 6 h after ALA injection (Fig. 6b). Fluorescence was no longer detected in the normal brain at 8 h. The fluorescence distribution in the contralateral brain did not differ for the two tumour models. Some fluorescence was visible in the pia mater and in the ipsilateral white matter. In the contralateral white matter fluorescence extended along

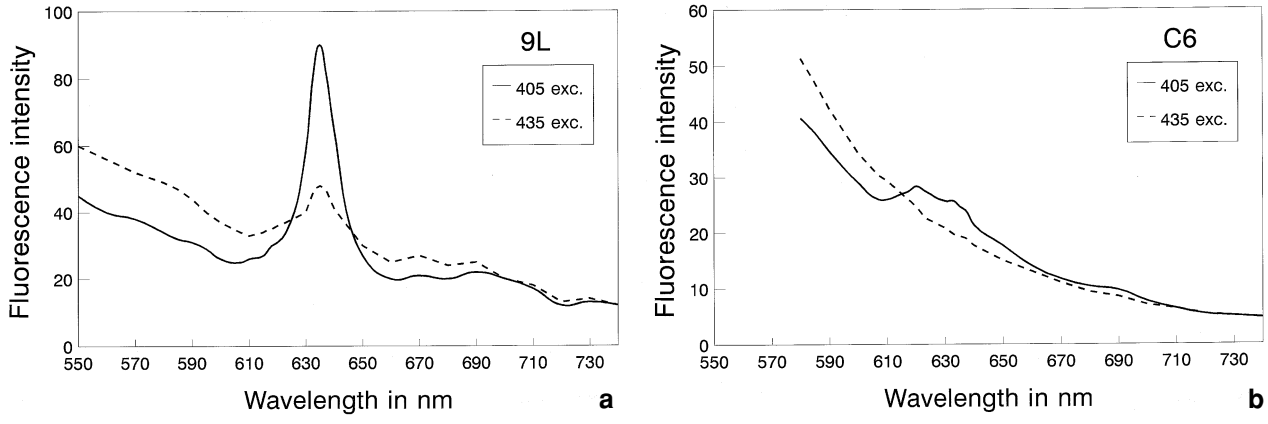

Fig. 5. Example of fluorescence emission spectra of intracranial tumours 2 h after administration of 200 mg kg^{-1} ALA. (a) 9L gliosarcoma, (b) C6 glioma

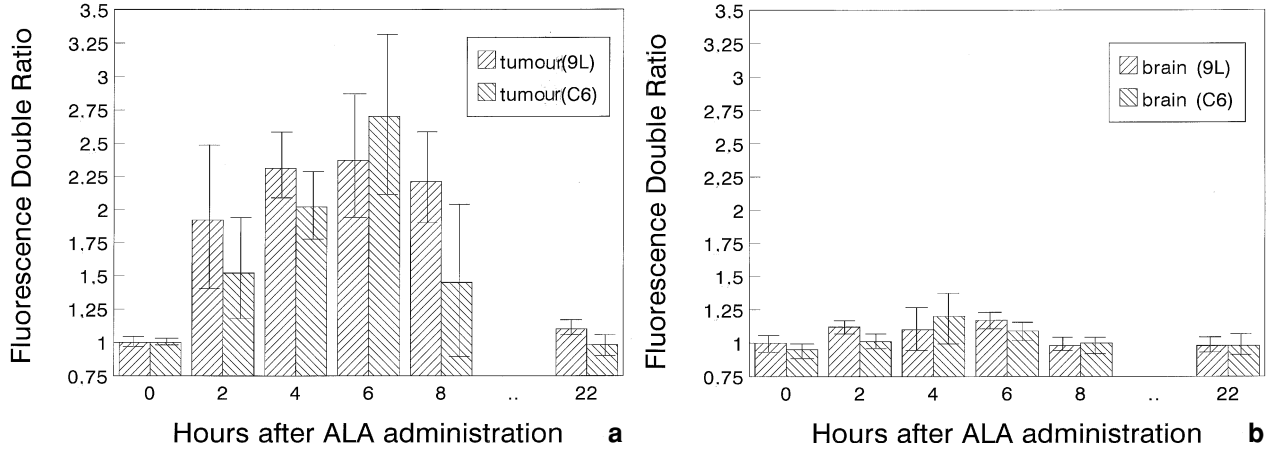

Fig. 6. Double ratio fluorescence $DR_{(635\,nm)}$ 9L and C6 tumours 0–22 h after i.v. administration of 200 mg kg^{-1} ALA. (a) Tumour, (b) contralateral brain. Note that zero porphyrin concentration corresponds with a DR of 1

the corpus callosum for about 1 mm. At 4 h the fluorescence in the normal brain surrounding the tumours was increased mainly along the blood vessels. At 6 h the most extensive fluorescence was seen in the ipsilateral white matter and the pia mater. In one animal the complete ventricle lining showed fluorescence, except for the plexus. At 8 and 22 h only faint fluorescence of the white matter tracts and some blood vessels in the tumour vicinity was seen. In contrast with the diffuse fluorescence in the hours before, now single slightly fluorescent cells were identified in the white matter. Groups of weakly fluorescent cells along larger vessels, probably macrophages, were the only other fluorescent structures found in the brain.

In the hemisphere with the cryo-lesion an increase of the fluorescence at 635 nm was measured. Only a slight fluorescence was seen in the subcortical white matter, comparable to the contralateral brain of the tumour bearing animals. In the contralateral hemisphere of the cryo-lesion no fluorescence was detected (Fig. 8).

No fluorescence was detected in the normal brain of the 5 control rats without a tumour, 6 h after injection of ALA. The same was true for all control rats with tumours without ALA.

Discussion

ALA-PDT has several advantages over the use of thus far available porphyrin photosensitizers. One advantage of the use of ALA-induced porphyrins over exogenous administered photosensitizers is their location in the cell. Since most porphyrins are lipophylic, after systemic administration they locate preferentially in the cell membrane [41] and at longer incubation

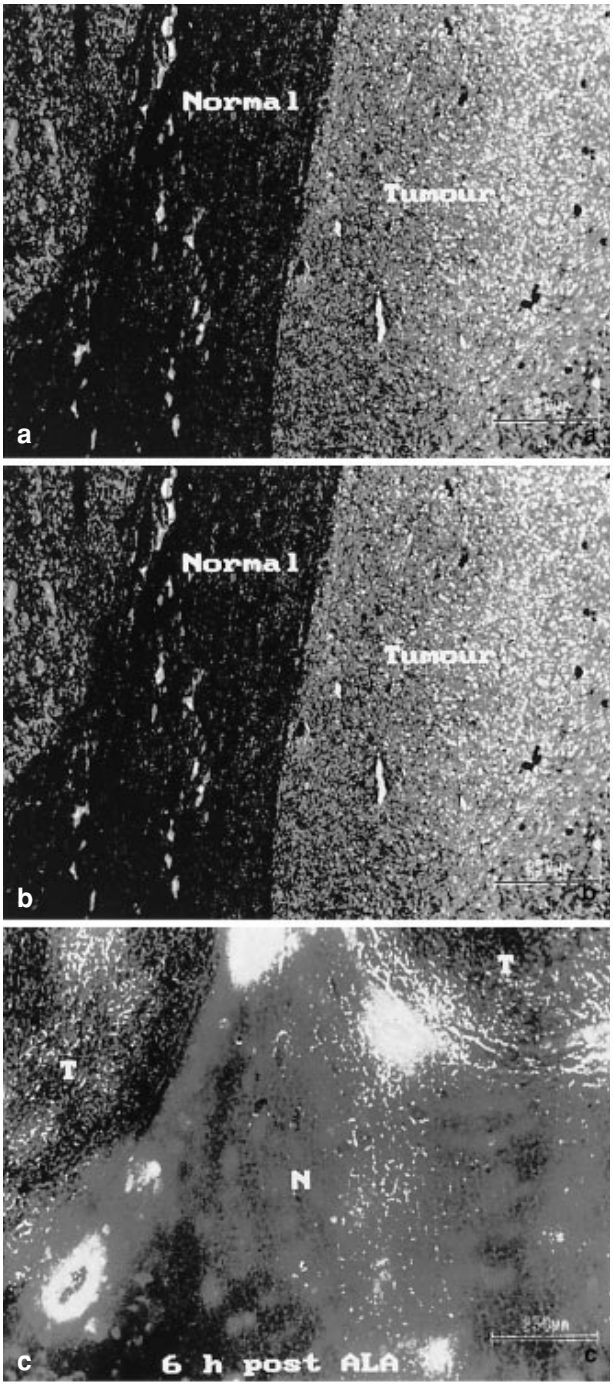

Fig. 7. (a) CLSM image of two 9L tumour lobes and several highly fluorescent perivascular tumour extensions 6 h after ALA administration. (b) CLSM image of a 9L tumour and the surrounding brain tissue 2 h after ALA administration. Several fluorescent small blood vessels are visible in the tumour and the adjacent white matter. (c) CLSM image of a C6 tumour 4 h after ALA administration. Necrotic areas are non-fluorescent. A blood vessel in the tumour is accentuated by fluorescence

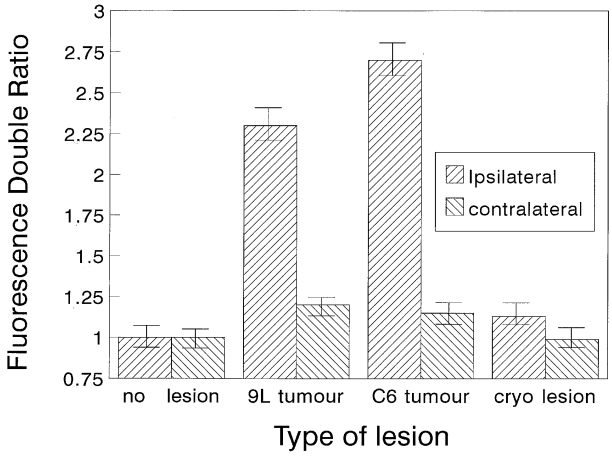

Fig. 8. A comparison between fluorescence $DR_{(635\,nm)}$ 6 h after ALA in the ipsi- and contralateral brain of the cryo-lesions, the tumours, and the brain of rats without a tumour

periods in membranous organelles [4, 46]. Besides PDT induced damage to the cell membrane, damage to mitochondria is seen as a crucial event preceding cell death [5, 43]. Endogenous PpIX is formed within the mitochondria. Initial damage after irradiation is therefore most likely to occur in these vital structures [21], which could make PDT with endogenous porphyrins more effective than PDT with exogeneously administered porphyrins.

ALA is a small, water soluble molecule. Its ability to pass the blood-brain barrier is still subject to dispute [2]. Terr and Weiner concluded that the BBB is virtually impermeable for ALA after i.v. injection of ^{14}C-labelled ALA to study the distribution of ALA within the brain [52]. Shortly after injection they found the ^{14}C-label only in areas of the brain lacking a barrier function, and in the CSF. In contrast, McGillion reported blood level dependent ALA concentrations in rat brain homogenates after administration [33, 34]. ALA extraction after brain tissue homogenisation does, however, not discriminate between areas with a lacking or an intact BBB, and also ALA in the vascular or CSF compartment could have contributed to the overall concentration.

The available information on the subsequent formation of endogenous porphyrins within the brain after systemic ALA administration is very limited. A porphyrin level rise from 0.08 to 1.09 nmol g^{-1} was reported in mice brain homogenates 6 h after injection of 2000 mg kg^{-1} ALA [1]. Sroka et al. detected porphyrin fluorescence in mice brain samples with a maximum at 1 h after injection of 50 mg ALA kg^{-1} [48].

Fukuda et al. injected ALA subcutaneously and intraperitoneally into mice [14]. In the first hours after injection of ALA a slight increase in porphyrin content of the brain occurred. The concentration doubled after injection of the same amount of ALA incorporated into liposomes, indicating that the uptake in the brain from lipophylic carriers is much more effective than that of the free ALA.

The Nature of the Porphyrins

In vivo, in the tumours both 595, 625, 635, 655 and 705 nm were peaks. Emission around 635 and 705 nm after ALA administration has been described in several tissues [3, 15, 17, 28, 31], and has been shown to represent PpIX [38]. Peaks around 625 nm are also well known, and were assigned to coproporphyrin (COPRO) [21, 28]. After ALA administration usually PpIX is assumed to accumulate in the tumour because of its central position in the biosynthesis of haem (Fig. 1). PpIX accumulation was indeed confirmed by high performance liquid chromatography for an oral squamous cell carcinoma in a patient who had received 60 mg/kg ALA per os 4 h prior to biopsy [17]. In this tumour more than 96% of the porphyrins consisted of PpIX. Fukuda et al. found a different composition of porphyrins in subcutaneous mammary carcinoma in mice at chromatography after ALA administration: PpIX constituted 35% of the porphyrins, while 45% was identified as COPRO and 4.3% as uroporphyrin (URO) [14]. Also erythroleucaemic cells are able to produce large amounts of URO and COPRO after incubation with ALA [32]. Two different patterns of porphyrin induction by ALA were demonstrated by Fritsch et al., who found mainly PpIX in human basal cell carcinoma, while kerato-acanthoma produced large amounts of COPRO [Fritsch, personal communication]. Indications for an aberrant porphyrin pathway with the accumulation of porphyrins other than PpIX come from a report of the natural accumulation of porphyrins in some tumours. Rubino et al. recovered only 10–15% PpIX from human lung tumours, while the larger part was mainly coproporphyrin and a trace of uroporphyrin [42]. This indicates that at least some tumours are able to make other porphyrins besides PpIX. Our data suggest that both tumours in vivo made other porphyrins besides PpIX, probably COPRO.

In vitro, the red fluorescence peak was strongest at excitation wavelengths of 405, 495, 530 and 575 nm. This resembles the typical porphyrin excitation peaks described in vitro and in vivo by other author [18, 21, 28, 38]. The emission spectra of the 9L and the C6 cells suggest that possibly more than one porphyrin species is formed. In contrast to the implanted tumours no difference was seen, however, in the spectra of the two cell cultures. This supports the hypothesis that the differences observed in vivo are related to changes in the in vivo metabolism due to environmental factors, as such environmental factors were identical in the in vitro experiments. The main fluorescence peak at 635 nm is likely to represent PpIX, 625 nm emission suggests the presence of COPRO. Peaks between 575 and 595 nm have been reported by several authors before [8, 27] and are likely to belong to Zn-protoporphyrin [10]. This species can be formed in vitro from traces of metallic ions in disposable plastics [47]. The 655 nm peak in the cell cultures is more difficult to explain, but could belong to porphyrin aggregates [9, 26, 35].

The Origin of the Porphyrins in Normal Brain Tissue

No porphyrins were detectable in the normal caudate nucleus 6 h after ALA administration in rats without a tumour. The results of our study support the view that has emerged from the literature of a very low direct penetration of ALA into the intact brain at best. This does not necessarily contradict the few reports of elevated ALA and porphyrin concentrations in the brain, since they were all based on homogenisation of brain samples and often of whole brains [1, 14, 33, 34]. This makes overestimation likely due to inclusion of tissues expected to yield ALA and accumulate porphyrins, for instance areas lacking a BBB, the blood, and the CSF. Uptake of porphyrins from the blood occurs in many tissues but can be ruled out in brain with intact BBB [23, 55]. Disruption of the BBB does facilitate the uptake of porphyrins from the blood into the surrounding normal brain [50, 55], and may explain our discovery of porphyrins in rats with a cortical cryo-lesion. A local production of porphyrins in or around the lesion seems unlikely because of the cell damage induced by the freezing. In contrast to the situation in rat brain without tumours, a large portion of the porphyrins which were detected in the normal brain tissue of tumour bearing rats in our study may have been produced in the tumours and redistributed into the surrounding brain. A continuous, but with distance decreasing red fluorescence, followed the white matter tracts from the tumours to the periphery

and the contralateral hemisphere. Transportation by convection of large molecules, for example serum albumin, originating from an experimental tumour or a cryogenic lesion has been shown over a wide area in the normal white matter [20, 40]. Bulk flow from the tumour hemisphere with the interstitial fluid current in the white matter of the brain is likely to facilitate the spread of porphyrins, which have a high affinity for serum proteins. Redistribution of porphyrins together with FITC-labelled albumin over a distance of 5 mm within 4 h was measured by Summer in the cortex of cats with a cryo-lesion [50]. Evidence strongly supporting a spread of tumour borne porphyrins to the normal brain is found in figure 8. There, the normal brain of rats without a tumour, with a cryo lesion shows no elevated porphyrin levels contralaterally. Thus the porphyrins in the normal brain seem to reflect the production in the tumours, rather than systemically circulating porphyrins produced in the liver.

ALA and Porphyrin Circulation in Blood and CSF

Porphyrins were detected after 2 to 6 h in the lining of some blood vessels in the brain near the tumour. It is not clear whether the porphyrins were formed within the endothelial cells or were taken up by the endothelial cells from the blood or from the perivascular space. Grant et al. studied ALA-induced PpIX in small arteries and veins in rats and found PpIX mainly in the endothelial lining of the vessels, with a peak at 3 h [19]. Egger et al. found high levels of circulating porphyrins in dogs during 10 h after a single administration of 100 mg kg^{-1} ALA [Egger, N. and Anderson, K. personal communication], thus enabling the endothelial cells to accumulate porphyrins from the blood stream as bovine endothelial cells have been found to do in vitro [16].

An additional source of porphyrins or ALA in the brain could be the CSF. In a patient with an attack of acute intermittent porphyria, a disease with high plasma levels of ALA, Sweeney et al. measured an extremely high level of ALA in the CSF [51]. The origin of this ALA remained obscure. In the case of a brain tumour, animal studies suggest the possibility of leakage from the blood through the defective BBB, and subsequent transportation via the interstitial fluid current directly to the lateral ventricles. Reulen et al. were able to demonstrate this route with labelled oedema fluid originating from a cortical cryo-lesion [40]. There is no barrier to diffusion between the CSF and the surrounding brain, and ALA is readily taken up and metabolised in the brain after intraventricular ^{14}C-ALA injection [44].

We found a strong fluorescence in the pia mater and ventricle lining at 6 h after ALA administration in tumour bearing rats. The fact that the plexus was non-fluorescent at the time of widespread ventricular fluorescence suggests that the porphyrins were not blood borne. It does, however, not discriminate between uptake of porphyrins from the CSF or local production of porphyrins from ALA in the ventricle lining ependym.

Porphyrin Induction in Different Tumours

The rapid production of porphyrins in vivo, starting at two hours and peaking around 6 hours, suggest that penetration and distribution of ALA into the tumours must have been fast. Although the overall porphyrin levels were comparable for 9L and C6 tumours, there was a difference in fluorescence distribution in the tumours. In the 9L tumour the infiltrating tumour had a very high fluorescence level and the pattern throughout the tumour was rather homogeneous, while in the C6 tumours the tumour edge had often low levels of fluorescence and in the tumours fluorescent areas intermingled with non-fluorescent parts. Since the porphyrin production level and kinetics were comparable for both tumours in vitro, an explanation could be found in the distinct histological characteristics of both tumours. Extensive perivascular infiltrations are a distinct characteristic of the 9L tumour and are found only sporadically in C6 tumours. In these tumours necrosis was a common feature, that was not seen in 9L tumours. This difference in growth pattern between both tumour models has not only consequences for the extracellular environment and metabolism as mentioned before, but is also related to the blood supply, as was shown by blood flow measurements in vivo [12, 13]. In 9L tumours the highest blood flow values were seen in brain regions with extensive perivascular infiltration (1.47 ml $gr.^{-1}$ min^{-1}), the lowest in the tumour centre (0.62 ml $gr.^{-1}$ min^{-1}) [13]. The blood flow in the viable C6 tumour tissue was as low as that of the 9L tumour centre (0.68 ml $gr.^{-1}$ min^{-1}) [13]. The high porphyrin fluorescence of the perivascularly infiltrating tumour can therefore not completely be explained by an elevated (haem) metabolism, but also suggests a very high access to ALA from the blood. The different fluorescence pattern of these histologically distinct tumours

yields a warning for the clinical application of ALA-induced porphyrin for PDT. Probably astrocytoma will behave differently from oligodendroglioma, and grade II tumours could be completely incomparable to grade III or IV tumours. Therefore the next step will have to be the evaluation of porphyrin synthesis in different human brain tumours in vivo.

Conclusion

Two rat brain tumour models were shown able to accumulate porphyrins in vitro and in vivo for several hours after administration of ALA. The slight rise of porphyrin fluorescence in the normal brain of tumour bearing rats, compared to no rise in rats without a tumour, is attributed to transport by bulk flow of porphyrins made in the tumours, and partly to systemic porphyrins leaking from the tumour vessels. By 22 hours almost no fluorescence was detected in tumour and brain.

In order to evaluate the usefulness of ALA-induced porphyrins for PDT of brain tumours, further important questions will have to be answered. Fluorescence studies in patients will have to show whether human brain tumours make sufficient porphyrins for PDT, whether infiltrating tumour cells can be reached, and whether damage to blood vessels in the tumour and/or the brain occurs.

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Comment

Hebeda *et al.* describe a series of experiments in which they try to circumvent two major problems with the administration of haematoporphyrin derived substances for photodynamic therapy: selective enrichement in tumour tissue and accumulation in the cell membrane. In their experiements they try to raise the levels of endogenous porphyrins by intravenous administration of 5-Aminolevulinic Acid which is capable of permeating into tumour tissue in the presence of blood brain barrier leakage. The substance raises endogenous porphyrin levels and causes intracytoplasmatic porphyrin levels to rise which has an advantage over the accumulation of porphyrins in the membrane which occurs with the administration of haemato-

porphyrin derivatives. They show that the accumulation in two different tumour models is different, thus emphasizing the variability of tumour physiology. Of particular interest is the finding, that the differential induction of different porphyrins in 9L and C6.

This is a nice study in which all the relevant experiments and respective controls have been performed. Especially the problem of ''collateral damage'' through bulk flow is nicely included. It can be concluded from the comparative experiments between C6 and 9L that also this therapy depends on the individual tumour physiology.

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